Optimization of lactic acid production by *Lactobacillus plantarum* strain Hui1 in a medium containing sugar cane molasses

Optimización de la producción de ácido láctico por *Lactobacillus plantarum* cepa Hui1 en un medio que contiene melaza de caña

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ABSTRACT

The aim of this study was to optimize lactic acid production by a native strain (Hui1) of *Lactobacillus plantarum* isolated from a Peruvian Amazon fruit (*Genipa americana*) in a medium supplemented with an agroindustrial by-product such as sugar cane molasses. Optimization was performed though one-factor-at-a-time studies followed by the Placket-Burman and central composite designs. The data were analyzed by using the Statistica® 10 software. Several carbon, nitrogen and ion sources were tested, and the optimum concentration of lactic acid achieved was 84.2 g L^{-1} in a medium containing as follows (in g L^{-1}): meat extract, 18.69; tryptone, 7.88; sugar cane molasses, 140; calcium carbonate, 15; dipotassium phosphate, 1; manganese phosphate, 0.03; sodium acetate, 5, and magnesium sulphate, 0.2. In addition, a high degree of conversion from sugar cane molasses to lactic acid was obtained ($Y_{p/s}$ 0.898 g g^{-1}). These results indicate the potential of *Lactobacillus plantarum* strain Hui1 to produce lactic acid in a medium supplemented with sugar cane molasses, an underutilized industrial by-product.

Key words: fermentation, response surface methodology, by-products, industry.

RESUMEN

El objetivo de este estudio fue optimizar la producción de ácido láctico por una cepa nativa (Hui1) de *Lactobacillus plantarum*, aislada de un fruto de la Amazonía peruana (*Genipa americana*), en un medio suplementado con un subproducto agroindustrial como la melaza de caña de azúcar. La optimización se realizó mediante el estudio de un factor a la vez seguido de los diseños de Placket-Burman y compuesto central. Los datos se analizaron utilizando el software Statistica® 10. Se evaluaron varias fuentes de carbono, nitrógeno y iones, alcanzándose una producción de ácido láctico de 84.2 g L^{-1} en un medio que contenía (en g L^{-1}): extracto de carne, 18.69; triptona, 7.88; melaza de caña de azúcar, 140; carbonato de calcio, 15; fosfato dipotásico, 1; fosfato de manganeso, 0.03; acetato de sodio, 5; y sulfato de magnesio, 0.2. Además, se obtuvo un alto grado de conversión de melaza de caña de azúcar a ácido láctico ($Y_{p/s}$ 0.898 g g^{-1}). Estos resultados indican el potencial de la cepa de *Lactobacillus plantarum* Hui1 para producir ácido láctico en un medio suplementado con melaza de caña de azúcar, un subproducto industrial subutilizado.

Palabras clave: fermentación, metodología de superficie de respuesta, subproductos, industria.

Introduction

Lactic acid (2-hydroxypropanoic acid) has multiple applications in the pharmaceutical, food, and chemistry industries as a food additive, pH regulator, and poly lactic acid precursor, among others (De Oliveira, Coelho, *et al*., 2018). In the last few years, the use of lactic acid has notably increased, and around 30% of the production is used for the development of biopolymers (Sengupta *et al*., 2020).

Lactic acid can be produced by chemical synthesis and microbial fermentation. The chemical pathway gives as a result an equal concentration of the racemic mixture (L and D stereoisomers) (De Oliveira, Coelho, *et al*., 2018). Fermentation, depending on the microorganism, results in the production of either one of the stereoisomers or in a variable composition of the mixture. Moreover, 90% of the lactic acid production worldwide is carried out by fermentation since it is more cost effective (De Lima *et al*., 2009; Coelho *et al*., 2011).

The genus *Lactobacillus* comprises a group of bacteria widely distributed in nature. This genus is characterized by its high tolerance to acidic pH values and its capacity to

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produce important concentrations of lactic acid, especially *Lactobacillus plantarum* (Hwang *et al.*, 2012; Behera *et al.*, 2018). However, *Lactobacillus plantarum* is an heterofermentative microorganism and requires a complex medium with several nutrients, including fermentable carbohydrates, vitamins, amino acids and minerals (Coelho *et al.*, 2011). During lactic acid production by this bacterium, the selection of carbon and nitrogen sources in the medium formulation is fundamental (Brinques *et al.*, 2010).

In recent years, lactic acid production using a number of industrial by-products from banana, corn, potato, wheat, etc., has been reported (Mufidah *et al.*, 2017). In this context, sugar cane molasses, a by-product from the sugar industry formed by fermentable sugars (sucrose, glucose and fructose, 50% w/v), nitrogen (0.5-0.9% w/v) and minerals, could be used as a substrate for the production of this compound (Coelho *et al.*, 2011). Sugar cane molasses is typically used as a low-cost animal food. However, it is also used to produce ethanol and has great potential in the production of chemicals (Dotaniya *et al.*, 2016). The production of this raw material in countries such as China is around 3 million t per year (Sun *et al.*, 2019), which makes it available for use in different industrial bioprocesses in a circular economy context.

The aim of this study was to optimize the lactic acid production by *Lactobacillus plantarum* strain Hui1 isolated from a Peruvian Amazon fruit (*Genipa americana*), in a medium supplemented with sugar cane molasses. Different components were tested, and optimization was achieved by the response surface methodology (RSM).

Materials and methods

Inoculum and culture preparation

Lactobacillus plantarum strain Hui1 was isolated from huito (*Genipa americana*), a fruit from the Peruvian Amazon. This strain was isolated and identified by our research group (Laboratorio de Biologia Molecular, Facultad de Farmacia y Bioquimica, Universidad Nacional Mayor de San Marcos) through partial sequencing of 16S rDNA (GenBank accession number: KY977384). The stock cultures were maintained in De Man, Rogosa and Sharpe (MRS) medium supplemented with 20% (v/v) glycerol at -20°C. The MRS medium composition was as follows (in g L^{-1}): glucose, 20; peptone, 10; yeast extract, 5; meat extract, 10; sodium acetate, 5; ammonium citrate, 2; dipotassium phosphate, 5; magnesium sulphate heptahydrate, 0.1, and manganese sulphate tetrahydrate, 0.05.

The pre-inoculum was grown in the MRS medium at 30°C for 24 h. Then, the inoculum was prepared by transferring 2 ml of the pre-inoculum into an Erlenmeyer flask containing 20 ml of MRS medium. The culture was incubated for 10 h at 37°C and then the cell concentration was determined by the correlation between the optical density (OD) and the cell density ($1x10^8$ colony-forming units (CFU) ml^{-1}) (McFarland curve). The cells were concentrated by centrifugation at 2000 *g* for 10 min, then washed twice with 50 mM phosphate buffer at pH 7.0, and finally re-suspended in 1 ml of the same buffer. The OD measurements were carried out at 620 nm. The inoculum cell concentration used in this work was 10^9 CFU ml^{-1}. All the fermentations were carried out in 250 ml flasks containing 50 ml of culture medium as the final volume.

Sugar cane molasses pre-treatment

Sugar cane molasses were obtained from the company Ajinomoto Peru S.A. Sugar cane molasses was heated at 70°C for 10 min in a water bath and filtered through Whatman filter paper of 11 µm. Subsequently, the concentration of total sugars was analyzed by the modified Anthrone method described by Rodríguez (1987). A concentration of 100 g of total sugars per liter of culture medium (100 g TS L^{-1}) was equivalent to 140 g L^{-1} of sugar cane molasses.

One-factor-at-a-time

The effect of the following 16 factors on the lactic acid production by *Lactobacillus plantarum* strain Hui1 was evaluated independently as follows: fructose, galactose, glucose, lactose, maltose, cellobiose, sucrose, Tween 80, sodium acetate, ammonium citrate, starch, and sugar cane molasses as carbon sources, as well as meat extract, yeast extract, casein tryptone, and bacteriological tryptone as nitrogen sources. This was done to decide the significant factors in lactic acid production. For these assays, 2.6 g L^{-1} of MRS medium was supplemented either with 12 g L^{-1} of each carbon source or with 4 g L^{-1} of each nitrogen source (Chauhan *et al.*, 2007). Additionally, the medium containing each nitrogen source was also supplemented with glucose (12 g L^{-1}). In the case of the media with carbon sources such as ammonium citrate and sodium acetate, they were also supplemented with glucose (12 g L^{-1}). After fermentation, the lactic acid concentration was determined by acid-base titration.

Effect of calcium carbonate

To determine the effect of calcium carbonate on the lactic acid production, 2.6 g L^{-1} of MRS medium was supplemented with 140 g L^{-1} of sugar cane molasses and 15 g L^{-1}

of calcium carbonate (Dumbrepatil *et al*., 2008; Pejin *et al*., 2015). The pH of the medium was adjusted to 6.5 with 1 M NaOH and the culture was incubated at 37°C for 5 d at 50 *g*. Samples were collected at 22, 46, 72, 94 and 118 h of fermentation. The concentration of lactic acid was determined using the modified ferric chloride method (Pacios *et al*., 2009).

Plackett-Burman design

The four significant factors from the one-factor-at-a-time study, as well as the effect of ions, including Mg^{2+}, Mn^{2+} and K^{1+} (magnesium sulphate, manganese sulphate and dipotassium phosphate) were analyzed through a Plackett-Burman design that was divided into two levels with two central points and a replicate (Tab. 1). Cultures were carried out in 25 ml flasks containing 20 ml of medium supplemented with 140 g L^{-1} of sugar cane molasses and 15 g L^{-1} of calcium carbonate (Chauhan *et al*., 2007; Coelho *et al*., 2011). The pH of the medium was adjusted to 6.5 and the cultures were incubated at 37°C for 5 d and 50 *g*. Data obtained were analyzed by using the Statistica® 10 software.

TABLE 1. Plackett-Burman design for lactic acid production by *Lactobacillus plantarum* strain Hui1.

Independent variables	Low level	Central level	High level
	g L^{-1}		
	-1	0	+1
Meat extract	1.0	5.5	10.0
Casein tryptone	1.0	5.5	10.0
Ammonium citrate	0.0	1.0	2.0
Sodium acetate	0.0	2.5	5.0
Magnesium sulphate	0.0	0.1	0.2
Manganese sulphate	0.0	0.025	0.05
Dipotassium phosphate	0.0	1.0	2.0

Central composite design

The two significant factors from the Placket-Burman design, the meat extract and the casein tryptone, were optimized by applying the central composite design (CCD) methodology at five levels, including five replicates for the central point (Tab. 2). A total of 13 experiments were carried out in 25 ml flasks containing 20 ml of medium with the following composition (in g L^{-1}): sugar cane molasses, 140; calcium carbonate, 15; dipotassium phosphate, 1; manganese sulphate, 0.03; sodium acetate, 5; and magnesium sulphate, 0.1. The medium was adjusted to a pH of 6.5, and the cultures were incubated at 37°C, for 5 d and 50 *g*. The model is represented by the quadratic Equation 1:

$$Y = \beta_0 + \Sigma\beta iXi + \Sigma\beta iiXi^2 + \Sigma\beta ijXiXj \quad (1)$$

where Y is the predicted lactic acid response in g L^{-1}, β_0 is the intercept, βii is the interaction coefficient, and the independent variables correspond to Xi and X_iX_j. According to Table 2, X_i corresponds to X_1 (meat extract in g L^{-1}) and X_j corresponds to X_2 (tryptone in g L^{-1}).

TABLE 2. Range and levels of the variables used in the central composite design.

Variable (g L^{-1})	Code	Range and levels				
		- 1.414*	-1	0	1	1.414*
Meat extract	X_1	12.34	14	18	22	23.66
Casein tryptone	X_2	5.38	6	7.5	9	9.62

*The values ±1.414 correspond to α obtained from √2.

The analysis of variance (ANOVA) was carried out and the values of the F-test, correlation coefficient (R), coefficient of determination (R^2), t-student and the lack-of-fit were determined. The results were obtained with a 95% confidence level (P<0.05). Then, three-dimensional response surface curves were plotted to assess the interaction among components. Finally, the optimum values of the medium components determined by RSM were tested experimentally, to validate the optimization methodology. Statistical analysis was performed using the Statistica® 10 software.

Analytical methods

Quantitative determination of lactic acid

After each fermentation of the media resulted from the one-factor-at-a-time, the Plackett-Burman, and central composite designs, the culture media were centrifuged at 4000 *g* for 5 min, recovering the supernatant liquid and discarding the pellet. The supernatants were analyzed by either of the two methods used for the quantitative determination of lactic acid. The first was the AOAC 947.05 method (AOAC, 2019). This was carried out as follows: one drop of phenolphthalein was added to 10 ml of the supernatant and then the mixture was titrated with 0.1 N NaOH. The lactic acid was calculated and expressed as g of lactic acid L^{-1} of culture. The second method was a colorimetric assay using ferric chloride (Pacios *et al*., 2009). In this method, 1.5% (w/v) calcium carbonate was added to the supernatant, and the mixture was heated to 50°C for 5 min. Then, this suspension was centrifuged at 2800 *g* for 10 min, and 1 ml of 61.6 mM ferric chloride was added to 1 ml of the recovered supernatant. The ferric lactate concentration was determined by spectrophotometry at 440 nm.

Biomass calculations

The biomass concentration was determined by turbidimetry diluting 0.5 ml of culture in 50 mM phosphate buffer at pH 7.0. The absorbance was measured at 620 nm and the cell concentration was determined by comparison with the OD and the cell density ($1x10^8$ CFU ml^{-1}) (McFarland curve).

Total sugars calculations

The total sugar concentration was determined using the modified Anthrone method (Rodríguez, 1987). The samples obtained from the fermentation were previously centrifuged at 4000 *g* for 5 min, and 500 ml of the supernatant were collected and placed in 2 ml microtubes on ice. Then, 1 ml of the Anthrone reagent was added. The mixture was subsequently homogenized and placed on ice; then, the tubes were transferred to a boiling water bath for 5 min. Afterwards, the tubes were placed on ice for 1 min. The measurement was carried out at a wavelength of 640 nm.

Determination of $Y_{p/s}$

The $Y_{p/s}$ is the yield of substrate in product, considering that P_0 (initial lactic acid in g L^{-1}), S_0 (initial total sugars in g L^{-1}), P_f (final lactic acid in g L^{-1}) and S_f (final total sugars in g L^{-1}) are the initial and final concentrations of product and substrate of the fermentation. It is the ratio of the lactic acid produced by consuming the total sugars present in the cane molasses in the fermentation medium (Eq. 2).

$$Y_{p/s} = \frac{P_f - P_o}{S_o - S_f} = \frac{Lactic\ acid\ produced\ (g)}{Total\ sugars\ consumed\ (g)} \quad (2)$$

Results and discussion

One-factor-at-a-time

This study was carried out to identify the carbon and nitrogen sources with a significant effect on the lactic acid production by the *Lactobacillus plantarum* strain Hui1. Figure 1 shows that all carbon sources were fermented except for starch. From these results, it can be observed that sugarcane molasses enhanced the lactic acid production 4.8-fold compared to the control (MRS medium). Molasses are low-cost agro-industrial by-products whose composition includes 40-60% sugars (approximately 11% reducing sugars, 34% sucrose), proteins, vitamins, and minerals (Sindhu *et al.*, 2016). The high sugar content, combined with their low market price, make them eligible to be used instead of synthetic sugars (glucose, fructose, and others). However, the composition of molasses will depend on different factors such as the variety of sugarcane, climate, and the process employed for sugar extraction (Sindhu *et al.*, 2016). The fermentation by *Lactobacillus plantarum* with molasses from different places (Komesu *et al.*, 2017), and sugarcane bagasse (Lino *et al.*, 2018), has a high potential in the production of lactic acid.

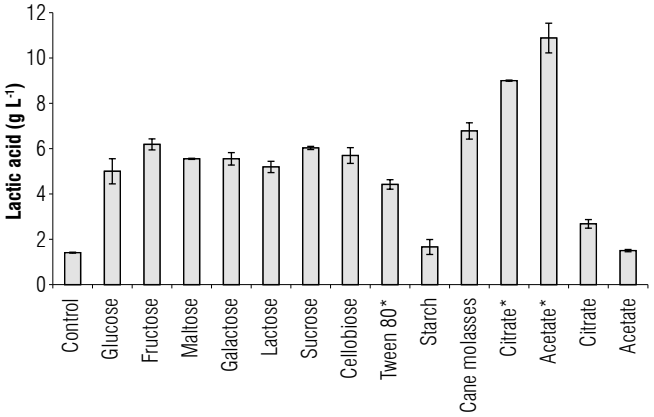

FIGURE 1. Effect of carbon sources on the lactic acid production by *Lactobacillus Plantarum* strain Hui1. *Supplemented with 12 g L^{-1} of glucose. Error bars represent the standard deviation from the mean (n = 3).

Figure 2 shows the effect of nitrogen sources on the production of lactic acid by the *Lactobacillus plantarum* strain Hui1. The greatest production of lactic acid was obtained with meat extract and tryptone; for this reason, they were selected for the Plackett-Burman design (Fig. 2). The nitrogen source plays a fundamental role on the growth of *Lactobacillus* strains. Several *Lactobacillus* strains are unable to synthesize some amino acids and, therefore, require a medium supplemented with them (Papizadeh *et al.*, 2020). Nitrogen from animal or bacterial origin represents an important source of these amino acids, low molecular weight peptides, and growth factors that protein hydrolysates provide. Safari *et al.* (2012) showed that *Lactobacillus plantarum* PTCC1058 had good growth in a medium containing peptones from the hydrolyzed wastes compared with the MRS control medium. They also indicated that *Lactobacillus plantarum* requires amino acids such as arginine, isoleucine, tyrosine, valine, and pantothenic acid for optimal growth. According to Solval *et al.* (2019), growth and the amount of lactic acid produced by *Lactobacillus plantarum* NRRL B-4496 in MRS medium without a nitrogen source (0.22 h^{-1}; 3.13 h and 1.73%) were lower than in the MRS control medium (0.48 h^{-1}; 1.44 h and 0.60%); thus, the absence of a nitrogen source affects the maximum growth rate, doubling time and lactic acid production.

The meat extract shows a higher concentration of vitamins, salts and approximately 57.83% of proteins in its

composition. On the other hand, tryptone contains a variety of essential amino acids with 13% of total nitrogen content in its composition. High nitrogen content allows a better growth of bacteria and, as a result, a greater production of lactic acid (Abedi & Hashemi, 2020).

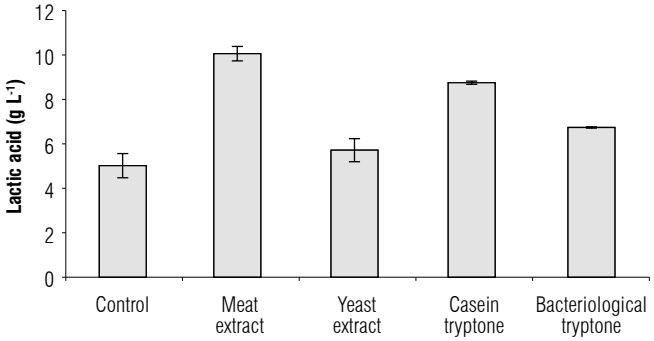

FIGURE 2. Effect of nitrogen sources on the lactic acid production by *Lactobacillus plantarum* strain Hui1. Error bars represent the standard deviation from the mean (n = 3).

Effect of calcium carbonate on lactic acid production

Figure 3 shows the effect of calcium carbonate on lactic acid production in the MRS medium supplemented with 140 g L^{-1} of sugar cane molasses. The maximum concentration of lactic acid was achieved at 72 h. Lactic acid concentration was remarkably higher in the presence of calcium carbonate than in the absence of this compound (3.5-fold). This could be explained due to the calcium carbonate regulating the pH of the culture during the fermentation process as it is considered a neutralizing agent (Yang *et al.*, 2015). According to Pejin *et al.* (2015), the *Lactobacillus* cells have a high viability due to the effect of calcium carbonate at the end of fermentation, and allow an increase in the lactic acid yield. Thus, Cubas-Cano *et al.* (2019) reported a lactic acid yield of 94% in a controlled pH fermentation, and a yield of 41% when pH was not controlled in the process.

Lactobacillus plantarum strains can function at low pH conditions (Iorizzo *et al.*, 2016); however, the acidification of the medium from a high accumulation of lactic acid causes an inhibition by-product that is not suitable for lactic acid overproduction (Singhvi *et al.*, 2018; Mousavi & Mousavi, 2019); also, the *Lactobacillus* growth is hampered by maintaining the transmembrane pH gradient (Othman *et al.*, 2017).

For these reasons, the use of calcium carbonate is necessary and, compared to other neutralizing agents (ammonia, sodium hydroxide, calcium hydroxide and potassium hydroxide), is a great alternative since it does not generate calcium sulphate as a by-product or cause cell toxicity by ammonia (Yang *et al.*, 2015).

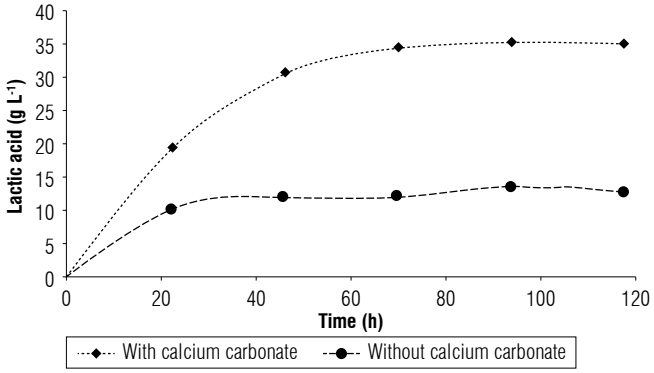

FIGURE 3. Effect of calcium carbonate on the lactic acid production from *Lactobacillus plantarum* Hui1.

The Plackett- Burman design

In this experiment, the concentrations of sugar cane molasses and calcium carbonate were kept constant at 140 and 15 g L^{-1}, respectively. Seven factors selected from the one-factor-a-time assays were tested, including meat extract and casein tryptone as nitrogen sources, ammonium citrate and sodium acetate as carbon sources, and Mg^{2+} (magnesium sulphate), Mn^{2+} (manganese sulphate) and K^{1+} (dipotassium phosphate) as ions. According to the ANOVA and the Pareto chart from the Plackett-Burman design (Tab. 3 and Fig. 4, respectively), the meat extract, casein tryptone, dipotassium phosphate, and manganese sulphate significantly influenced the lactic acid production (P<0.05). Both the meat extract and casein tryptone were important components in the culture medium due to their high concentration of peptides, amino acids, and vitamins. Therefore, as sugar cane molasses has a low organic nitrogen content, the concentrations of meat extract and casein tryptone used can be increased to improve lactic acid yields and the growth of *Lactobacillus plantarum* in a medium containing sugar cane molasses (Lino *et al.*, 2018; Papizadeh *et al.*, 2020).

TABLE 3. Analysis of variance (ANOVA) of the Plackett-Burman design for the lactic acid production by the *Lactobacillus plantarum* strain Hui1.

Factor	Sum of squares	Degrees of freedom	Square mean	F-value	*P*-value
Meat extract	816.41	1	816.41	134.15	0.0000[s]
Casein tryptone	179.92	1	179.92	29.56	0.0003[s]
Ammonium citrate	0.10	1	0.097	0.02	0.9019
Sodium acetate	24.46	1	24.46	4.02	0.0728
Magnesium sulphate	4.24	1	4.24	0.69	0.4233
Manganese sulphate	171.72	1	171.72	28.22	0.0003[s]
Dipotassium phosphate	142.95	1	142.95	23.49	0.0007[s]
Error	60.86	10	6.09		
Total sum of squares	1400.66	17			

Significance level: P<0.05; R^2: 0.956; R^2 (adjusted): 0.926, and s: significant at P<0.05.

Manganese sulphate showed a positive effect on the lactic acid production due to Mn^{2+} being a cofactor of several *Lactobacillus* enzymes such as the RNA polymerase, lactate dehydrogenase (LDH), NADH oxidase and superoxide dismutase that allows improving the efficiency and productivity of the lactic fermentation (Cheng *et al*., 2014). Cheng *et al*. (2014) demonstrate the effect of the Mn^{+2} on the stimulation of LDH by directing the conversion of pyruvic acid to lactic acid, and they show that the production of lactic acid decreases in the absence of Mn^{2+}. However, although *Lactobacillus plantarum* CCFM436 tolerates high concentrations of Mn^{2+}, its growth is affected (Tong *et al*., 2017). For that reason, it is necessary to adjust the Mn^{2+} concentration to prevent a negative effect on the lactic acid production. Although dipotassium phosphate has a negative effect on the lactic acid production, it is kept in the culture since KPO_4^{2-} is an inorganic salt important for the metabolism of *Lactobacillus plantarum* as it interacts with metal ions (Mn^{2+} and Mg^{2+}), thus improving the tolerance of bacteria to oxidative stress (Correa Deza *et al*., 2017; Alcántara *et al*., 2018). Therefore, the manganese sulphate and dipotassium phosphate concentrations were kept constant in the medium (0.03 and 1 g L^{-1}, respectively).

Regarding Mg^{2+}, the concentration of magnesium sulphate was kept constant at 0.2 g L^{-1} in the medium. Lew *et al*. (2012) indicate that the interaction between Mn^{2+} and Mg^{2+} is related to the obtention of good production of organic acids in *Lactobacillus*. In another study, Lew *et al*. (2013) indicate that both factors (Mn^{2+} and Mg^{2+}) exert a synergistic effect to achieve a high production of lactic acid; therefore, despite the fact that in our case magnesium was not significant, it was kept at a constant value in the following experiments.

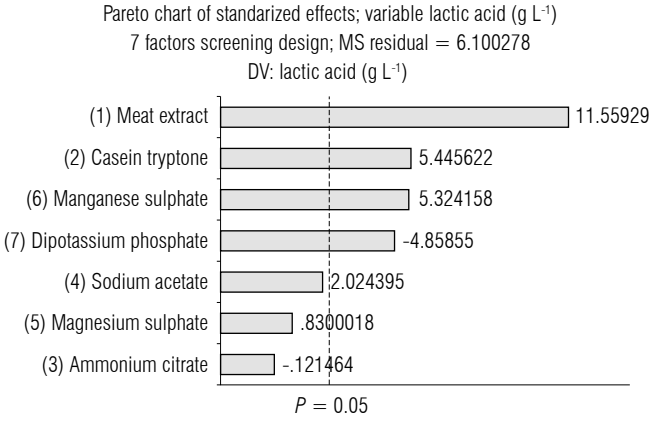

FIGURE 4. Pareto chart of the Placket-Burman design for the lactic acid production by *Lactobacillus plantarum* Hui1. MS residual - residual mean square and DV - dependent variable.

Central composite design

From the Placket-Burman design, the meat extract and casein tryptone were selected as the key factors for further optimization in lactic acid production by *Lactobacillus plantarum* Hui1. The ANOVA of the CCD (Tab. 4) shows that the lack of fit value was 2.94 and the *P*-value was not significant (0.07). According to this, the model fitted the data well, indicating that it can describe the influence of the factors on the response to predict lactic acid production.

TABLE 4. Analysis of variance (ANOVA) of the central composite design (CCD) for lactic acid production by the *Lactobacillus plantarum* strain Hui1.

Factor	Sum of squares	Degrees of freedom	Square mean	F-value	*P*-value
(1) Meat extract (L)	14.32	1	14.32	27.04	0.0001[s]
(2) Meat extract (Q)	106.36	1	106.36	200.81	0.0000[s]
(3) Casein tryptone (L)	6.74	1	6.74	12.73	0.0091[s]
(4) Casein tryptone (Q)	23.45	1	23.45	44.23	0.0002[s]
1L by 2L	0.02	1	0.02	0.03	0.8633
Residual	3.71	7	0.53		
Lack of fit	2.94	3	0.98	5.11	0.0745
Error	0.77	4	0.19		
Total sum of squares	143.59	12			

Significance level: $P<0.05$; $R^2 = 0.97$; R^2 (adjusted) = 0.95; L - linear; Q - quadratic; 1L by 2L: interaction between the two linear variables, and s: significant at $P<0.05$.

Equation 3 defines the fitted model, with the regression coefficients shown in Table 5. This quadratic model contains two linear terms, two quadratic terms and one factorial interaction.

$$Y = -48.0079 + 9.0512X_1 - 0.2444X_1^2 + 12.6581X_2 - 0.8161X_2^2 + 0.0108\,X_1\,X_2 \quad (3)$$

where Y is the predicted response (lactic acid concentration), and X_1 (meat extract) and X_2 (casein tryptone) are natural variables.

The meat extract and the casein tryptone had a significant effect ($P<0.05$) on the lactic acid production as well as positive coefficients (Tab. 5). Thus, an increase in the concentration of these compounds will result in an increase in lactic acid production. The interaction between the squared variables X_1^2 and X_2^2 was also significant ($P<0.05$), but their coefficients were negative, suggesting that at high concentrations of these factors (in relation to the quadratic form (Q) of the factors), obtaining lactic acid could be impaired. The X_1X_2 interaction was not significant ($P>0.05$). Additionally, the R^2 indicated that 97.42% of the variance could be explained by the quadratic model.

TABLE 5. Regression coefficients of the central composite design (CCD) for lactic acid production by the *Lactobacillus plantarum* strain Hui1.

Factor	Regression coefficients	Standard error	t (7)	*P*-value	-95% Confidence level	+95% Confidence level
Mean/Intercept	-48.0079	12.41799	-3.8660	0.0061^{s}	-77.3718	-18.6440
(1) Meat extract (L)	9.0512	0.77233	11.7194	0.0000^{s}	7.2249	10.8774
(2) Meat extract (Q)	-0.2444	0.01725	-14.1708	0.0000$_{s}$	-0.2852	-0.2036
(3) Casein tryptone (L)	12.6581	2.14595	5.8986	0.0006^{s}	7.5837	17.7324
(4) Casein tryptone (Q)	-0.8161	0.12264	-6.6545	0.0003^{s}	-1.1061	-0.5261
1L by 2L	0.0108	0.06065	0.1786	0.8633	-0.1326	0.1542

Significance level: $P<0.05$; L - linear; Q - quadratic; 1L by 2L: interaction between the two linear variables, and s: significant at $P<0.05$.

The graph of the response surface model (Fig. 5) showed that concentrations around 18 g L^{-1} of meat extract and 8 g L^{-1} of casein tryptone produced 86.23 g L^{-1} of lactic acid. Concentrations higher than 22 g L^{-1} of meat extract and 10 g L^{-1} of casein tryptone showed a reduction in the lactic acid production, demonstrating that nitrogen concentrations higher than 20 g L^{-1} were toxic for this *Lactobacillus plantarum* strain.

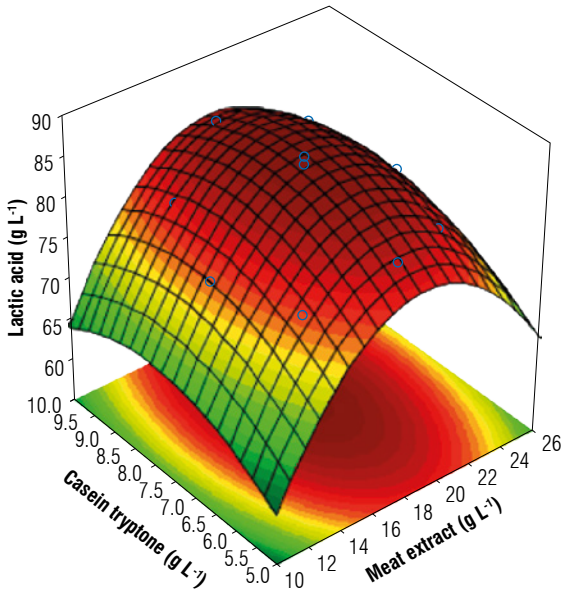

FIGURE 5. 3D response surface of the central composite design (CCD) showing the effects of the meat extract and casein tryptone on the lactic acid production by the *Lactobacillus plantarum* strain Hui1. MS pure error - mean square pure error and DV - dependent variable.

The maximum lactic acid production determined by CCD (86.44 g L^{-1}) was achieved using a medium containing 18.69 g L^{-1} of meat extract, 7.88 g L^{-1} of casein tryptone, 140 g L^{-1} of sugar cane molasses, 15 g L^{-1} of calcium carbonate, 1 g L^{-1} of dipotassium phosphate, 0.03 g L^{-1} of manganese sulphate, 5 g L^{-1} of sodium acetate and 0.2 g L^{-1} of magnesium sulphate. These results were tested and a lactic acid production of 84.2 g L^{-1} was obtained, demonstrating that the model was predictable.

Previous studies on the optimization of lactic acid production by different microorganisms are known (De Lima *et al.,* 2009, 41.42 g L^{-1}; Tripathi *et al.,* 2015, 39.2 g L^{-1}; Mufidah *et al.,* 2017, 28.01 g L^{-1}). In these studies, different industrial by-products were used, but the concentration of the lactic acid obtained was considerably lower than that in this study.

Other studies with *Lactobacillus plantarum* strains have shown that the nitrogen source improves the production of lactic acid, such is the case of Solval *et al.* (2019). They showed improved lactic acid production by *Lactobacillus plantarum* NRRL B-4496 in media containing high free amino acids and protein. Coghetto *et al.* (2016) obtained 21 g L^{-1} of lactic acid by *Lactobacillus plantarum* BL011 growing in soy bean acid residue (20 g L^{-1}), peptone (2 g L^{-1}), corn steep liquor (5 g L^{-1}), and yeast extract (15 g L^{-1}). These studies show the necessity for the fermentation medium to have a carbon source and a nitrogen source. Unban *et al.* (2019) found that gelatinized starchy waste and corn steep liquor were the two nutrients that significantly influence lactic acid production by *Lactobacillus plantarum* S21. Hence, corn steep liquor, a nitrogen source, could replace the meat extract and tryptone casein since these are considerably more expensive nitrogen sources in fermentation processes.

Some authors have worked using derived low-cost sugar media, such as Sikder *et al.* (2014), who reported a production of 89.5 g L^{-1} of lactic acid and $Y_{p/s}$ of 0.83 g g^{-1} by *Lactobacillus plantarum* NCIM 2912 starting with sugarcane juice (140 g L^{-1}) and yeast extract. This value is similar to that obtained in this study. Mousavi and Mousavi (2019) showed that lactic acid was the most significant product at the end of fermentation by *Lactobacillus plantarum* DSMZ 20174 using high fructose corn syrup. Zhang and Vadlani (2015) obtained a $Y_{p/s}$ of 0.87 g g^{-1} and 25 g L^{-1} of lactic acid by *Lactobacillus plantarum* ATCC 21028 from poplar hydrolysate (biomass-derived sugars).

In this case, molasses have been used as an efficient carbon source for the production of metabolites of industrial interest such as lactic acid (Papizadeh *et al*., 2020), with lower cost compared to sugarcane juice, since molasses is an industrial waste. Furthermore, as in the study of Coelho *et al.* (2011), supplementation with nitrogen sources was considered. These authors carried out the optimization of the medium composition in lactic acid production by *Lactobacillus plantarum* LMISM6 grown in molasses. A maximum lactic acid production of 94.8 g L^{-1} was obtained in a medium containing molasses, corn steep liquor, dipotassium phosphate and Tween 80 at concentrations of 193.50 g L^{-1}, 37.50 ml L^{-1}, 2.65 g L^{-1}, and 0.83 ml L^{-1}, respectively. Srivastava *et al.* (2014) showed a strong interaction between molasses and amino acids. High concentrations of nitrogen (>40 g L^{-1}) had a negative effect, while an increase in the concentration of cane molasses improved lactic acid production.

The $Y_{p/s}$ obtained at the end of the fermentation was 0.89 g g^{-1} and indicated that sugar cane molasses was a suitable substrate for lactic acid production by *Lactobacillus plantarum* strain Hui1. The production of lactic acid obtained in this study was similar to that achieved by De Oliveira, Rossell, *et al.* (2018) who obtained a $Y_{p/s}$ of 0.93 g g^{-1} by *Lactobacillus plantarum* CCT 3751 from hemicellulosic liquor from sugarcane bagasse, yeast extract (20 g L^{-1}), and sodium acetate (5 g L^{-1}).

Conclusions

The optimization of lactic acid production by *Lactobacillus plantarum* strain Hui1 was achieved by RSM obtaining a yield of 84.2 g L^{-1}. The results showed that *Lactobacillus plantarum* strain Hui1 has the potential to be used for the industrial production of lactic acid. In addition, this research demonstrated the feasibility of using an agro-industrial waste such as sugar cane molasses as a promising carbon source in the culture medium for lactic acid production. Finally, it is important to highlight that the replacement of synthetic sources with by-products in a culture medium contributes to reduce production costs. This work provides insights for continuing the search for low-cost sources for the medium culture formulation, mainly nitrogen sources.

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Author's contributions

SS conducted the research and investigation process, applied the statistical techniques to analyze the study data and developed the methodology. LAP formulated the overarching research goals and aims, conducted the research and investigation process, developed the methodology and verified the overall replication/reproducibility of results/experiments. JCFS conducted the research and investigation process, applied the statistical techniques to analyze the study data, and developed the methodology. CNFF formulated the overarching research goals and aims, conducted the research and investigation process, wrote the original draft, and carried out the critical review, commentary, or revision of the manuscript. HAG wrote the original draft and carried out the critical review, commentary, and revision of the manuscript. AIZP obtained the financial support for the project leading to this publication, managed and coordinated the research activity planning and execution, wrote the original draft, and carried out the critical review, commentary, and revision of the manuscript.

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